Effect of Aliphatic Alcohols on Bovine Alkaline Phosphatases

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Introduction

Aliphatic alcohols in high concentrations have been found to affect the activity of bovine alkaline phosphatases. Since the change in activity appeared to reflect the change in the dielectric properties of the solution, pH and concentration of substrate were varied, as well as alcohol concentration, to determine, if possible, the effect on the enzyme–substrate combination. The bovine alkaline phosphatase from milk and that from intestinal mucosa behaved differently with alcohol, which is further evidence that these enzymes are different entities (1).

Methods

The phosphatase from intestinal mucosa of the calf was the same as that used in previous experiments (2). Approximately 4 μg. was used in each assay. The phosphatase from cow's milk was prepared by acetone fractionation of preparations previously described (2). It was not adsorbed on Filter-Cel (3), and contained 4000 units/mg. Approximately 13 μg. was used in each assay.

The activity of the phosphatases was determined by the previously described method, except that the final volume was 13 ml. instead of 12 ml. (2); 8.1 ml. of 0.1 *M* ethanolamine·HCl buffer, 0.2 ml. of 0.15 *M* $MgCl_2$, 0.2–0.9 ml. of 0.01 *M* phenyl phosphate as substrate, 1.0 ml. of suitably diluted phosphatase, and water or alcohol to make the final volume 13.0 ml. were used in each assay. pH values were always determined on these mixtures, for the alcohols lowered the buffer values somewhat (i.e., the dissociation of the ethanolamine as a base was decreased), and when the buffer solutions were used at the extreme of the buffer range for ethanolamine (maximum buffering, pH 9.5), the addition of the other reagents lowered the pH values. The data are presented in terms of photometer readings, showing the phenol released in a hydrolysis period of 5 min. The presence of alcohol had no effect on the color given by the Folin-Ciocalteu reagent with phenol.

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Results

The inhibition of the intestinal phosphatase resulting from the presence of alcohol at 22° was identical whether the time of contact between phosphatase and alcohol in the assay mixtures was 2 or 30 min. Routinely, 2 min. elapsed before the substrate was added and the assay performed. From these results, it was concluded that the inhibitions were not a result of denaturation of the phosphatase by the alcohols but probably indicated an effect on the enzyme–substrate equilibrium. This conclusion is supported by the nature of the complete data obtained.

On a volume per cent basis, the three alcohols studied, ethanol, methanol, and isopropanol, over a range of concentration were equally inhibitory to the intestinal phosphatase in the standard assay at pH 9.70. The results obtained with ethanol at a concentration of substrate of 0.00077 *M* are shown in Fig. 1. Since in the 20% by volume solutions of ethanol, methanol, and isopropanol, the molar alcohol concentrations were 3.4, 5.0, and 2.6, respectively, it is believed the effect was not a molar one. An alteration in the total solvent environment such as the dielectric constant of the solution is the more likely explanation. Calculating from the values for the dielectric constants of water and of these alcohols given by Bull (4) it is found that the dielectric constants of the three 20% alcohol solutions differ by only 4%. The method of assay would not detect a difference of this magnitude. In contrast to the intestinal phosphatase, milk phosphatase at this pH and concentration of substrate is negligibly affected by the alcohols.

The most interesting results were obtained when the effect of ethanol was studied over a range of pH values. Figure 2 gives the results for the intestinal phosphatase for 0.00077 *M* (*A*) and 0.00017 *M* (*B*) substrate concentrations. The individual experiments from which these curves were prepared showed a range of ± 5%; most of the data were within half this range. With both concentrations of the substrate (*A* and *B*), inhibition was obtained but only on the acid side of the pH–activity curve.

The results obtained with the milk phosphatase over a range of pH values (Fig. 3) give a different picture. Ethanol stimulates the milk phosphatase but only on the basic side of the pH–activity curve, and the effect is greater with the higher concentration of the substrate. Measurement of the phosphate released during the hydrolysis showed

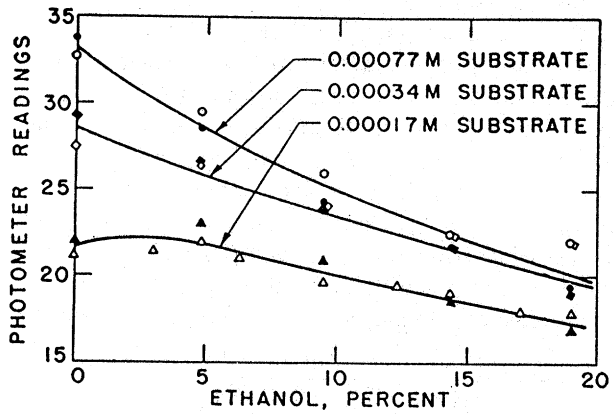

FIG. 1. Effect of ethanol at pH 9.7 on intestinal alkaline phosphatase in relation to concentration of substrate. Solid symbols indicate data read from pH–activity slopes; open symbols indicate data with buffer mixtures chosen to give pH 9.70 with ethanol present.

that it paralleled the release of phenol; hence a transphosphorylation is not involved as it is with the acid phosphatase (5,6).

The effect of ethanol on the intestinal alkaline phosphatase at pH 9.7 with several concentrations of substrate is presented in Fig. 1. Since the ethanol affected the pH of the buffer, mixtures of buffers were used to obtain a constant pH over the range of ethanol concen-

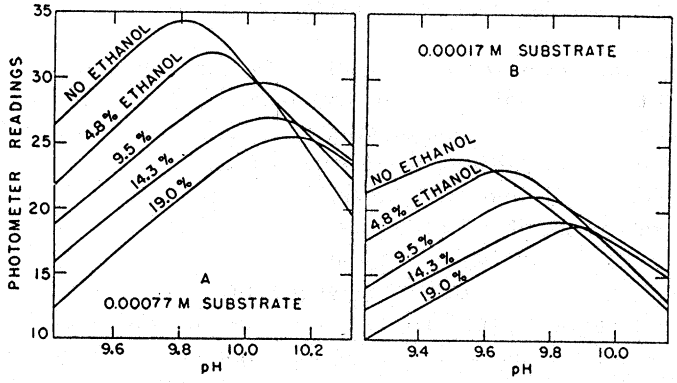

FIG. 2. Effect of ethanol on intestinal alkaline phosphatase in relation to pH with two concentrations of the substrate phenyl phosphate.

trations. In addition, measurements were made at several pH values, and the value for 9.7 was read from the pH slope. Because of the large effect of small variation in pH, the latter procedure probably gives the most reliable data. In addition to the data shown in Fig. 1, results were also obtained for 0.00060 and 0.00026 *M* substrate, but beyond the 5% ethanol concentration the curves obtained grouped with the two top curves shown. In the presence of the higher concentrations of ethanol, reduction in the concentration of substrate did not reduce the activity until the concentration was less than 0.00026 *M*. From this, it can be concluded that the combination of enzyme and substrate is enhanced in the presence of ethanol, that is, the value of the enzyme–

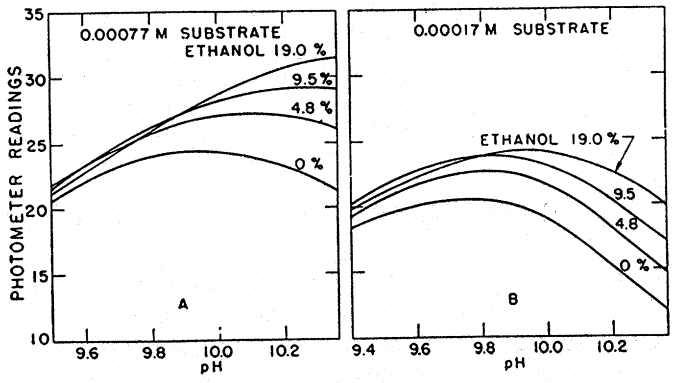

FIG. 3. Effect of ethanol on milk alkaline phosphatase in relation to pH with two concentrations of the substrate phenyl phosphate.

substrate equilibrium constant K_m is smaller. The assay method needs to be modified to increase the precision of the data and to permit the experiments to be extended to concentrations of substrate below 0.00017 *M* in order to obtain a quantitative measure of K_m.

It was considered possible that the shift of the pH optima of the phosphatases to higher values in the presence of ethanol might reflect an increase in the dissociation of the substrate (7) in this solvent. However, titration of monosodium phenyl phosphate with NaOH in the presence of ethanol showed that on the contrary the apparent dissociation was decreased; hence, this could not explain the shift in the pH optima. The shift of the pH optimum with concentration of the substrate has previously been observed by Neumann (8).

Cations are inhibitory to alkaline phosphatase (1,2) somewhat as shown by ethanol in Fig. 2 [inhibition of the acid side of the pH–activity curve and shift of the optimum along the basic side (9)]. Because of this similarity, experiments were performed with the cationic ethanolamine buffer at one-half the usual concentration, since it was believed that the ethanol might influence the ethanolamine–phosphatase equilibrium. This appears not to be the case, for the effects of ethanol on both the intestinal and milk phosphatases were the same with both buffer concentrations.

Discussion

Effects of alcohols on the activity of enzymes have been reported in a number of instances. Low concentrations of alcohols (<0.5 M) (10) activate unpurified tissue cholinesterases, and high concentrations of alcohol inactivate the enzyme. The first effect is rapid and reversible, whereas the latter is slow and irreversible. Since the activation is high with low concentrations of alcohol (as much as 100% with 0.4 M n-butanol) and the inactivation appears to be a denaturation, the effect probably is quite different from that observed with the phosphatases.

The effect of methanol on chymotrypsin, described by Kaufman and Neurath (11), appears to be most related to the results obtained in the present studies. The inhibition exerted by the methanol on chymotrypsin with various concentrations of the substrate acetyl-L-tyrosinamide showed that the enzyme–substrate equilibrium constant K_m increased, whereas the maximum velocity V_{max} remained constant. Kaufman and Neurath discuss their results in terms of the lowering of the dielectric constant of the solvent by alcohols, in relation to charge or polarity of specific sites on the enzyme and substrate, and formation of the enzyme–substrate combination. The increase in K_m in their studies where the substrate is uncharged, and the decrease in K_m observed when the alkaline phosphatases act on the charged substrate phenyl phosphate are consistent with the picture they present. In the case of the milk phosphatase, the decrease in K_m is apparent as an actual increase in the activity of the enzyme. In the case of the intestinal phosphatase, the V_{max}, or decomposition velocity of the enzyme–substrate complex to give the hydrolytic products, is decreased also with an over-all decrease in the activity of the enzyme.

Van Slyke and Zacharias (12) have stated that both phases of the action of urease on urea are affected by 30% ethanol. The maximum

velocity of hydrolysis (excess substrate present) is depressed 50%, whereas a term inversely related to K_m decreases only 29%. The authors concluded that ethanol interferes relatively little with the combination of urease and substrate.

When charged groups are involved in the combination of enzyme and substrate or the decomposition of the enzyme–substrate complex, the degree of dissociation of these groups is important in determining the enzyme activity. The change in the dissociation of these groups effected by alcohols consequently will be another factor determining the level of phosphatase activity. Differences in the degree of dissociation of specific charged groups could account for the different properties [see present paper, also Ref. (2)] of the intestinal and the milk phosphatases.

Summary

Ethanol, methanol, and isopropanol (5–20%) are inhibitory to bovine intestinal alkaline phosphatase at pH 9.7. The bovine milk phosphatase is not affected by alcohols at pH 9.7 but is stimulated at higher pH values. Studies over a range of pH values, and concentrations of ethanol and the substrate phenyl phosphate, suggest that the ethanol, by reducing the dielectric constant of the medium, influences the enzyme–substrate interaction both directly and through the dissociation of charged groups involved in phosphatase activity. Differences in the degree of dissociation of the specific charged groups could account for the different properties of the two phosphatases.

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